International Journal of Pharmaceutics, 22 (1984) 75–87
Elsevier

IJP 00746

Physicochemical and antitumor characteristics of some polyamino acid prodrugs of mitomycin C

C.F. Roos *, Satoshi Matsumoto, Yoshinobu Takakura, Mitsuru Hashida and Hitoshi Sezaki

Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto (Japan)

(Received April 25th, 1984)
(Modified version received and accepted June 22nd, 1984)

Summary

In order to obtain more information about the application of polyamino acids as high molecular weight carriers of mitomycin C (MMC), conjugates with the polyamino acids: poly-L-glutamic acid (PGA; mol. wt. 11,000 and 60,000), poly-L-aspartic acid (PAA; mol. wt. 14,000) and poly-L-lysine (PLY; mol. wt. 13,000) were synthesized. Some physicochemical and antitumor characteristics of these conjugates were investigated. Gel filtration confirmed covalent binding and provided information about the molecular sizes. The release rates of MMC from conjugates were determined in vitro. The PAA and PGA (mol. wt. 11,000) conjugates acted as negatively charged molecules in their interaction with ion exchangers. The PLY conjugate showed a positive charge and was able to bind to Ehrlich Ascites Carcinoma cells in vitro. The effects of 1 h exposure of mouse L1210 leukemia cells to the conjugates were evaluated using cell culture system. In this experiment, only the PLY conjugate showed better effects than MMC. Continuous exposure to the conjugates showed a similar effect to MMC. In vivo, less toxicity was found for the conjugates than for MMC. The PGA (mol. wt. 11,000) and PLY conjugates showed slightly higher effects against P388 leukemia than MMC, while no toxic doses were reached.

* Connected to Kyoto University as research-fellow from the State University Leiden, The Netherlands, Subfaculty of Pharmacy, Division of Pharmacology and Pharmacotherapeutics, Prof. D.D. Breimer.
Correspondence: H. Sezaki, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

Introduction

One of the limitations for the use of mitomycin C (MMC) in chemotherapy is its toxicity to bone marrow and intestine (Bradner, 1980). These side-effects might be reduced by concentrating the cytotoxicity at the tumor site (Sezaki and Hashida, 1984; Poznansky and Cleland, 1980). One possibility is to alter the biopharmaceutical properties of the drug, in this case by conjugation to a high molecular weight compound. Such conjugates could act as immobile or mobile molecular depots for the parent agent. This could be useful in the treatment of malignancies in isolated body cavities. Also other compounds that alter the biopharmaceutical properties of the conjugate, such as antibodies and lectins, could be attached to the high molecular weight back-bone (Sezaki and Hashida, 1984). These methods could lead to an increase of therapeutic effect at the desired site of action, while toxic and immunosuppressive side-effects are reduced. In previous papers, much research was done with polysaccharide conjugates of mitomycin C (Kojima et al., 1980; Hashida et al., 1981, 1984; Takakura et al., 1984) and other high molecular weight compounds. But little is known about the use of polyamino acids as high molecular weight back-bones because they may have some problems with regard to allergic reactions, purity and synthesis.

However, some polyamino acids have shown some interesting features. It was shown that poly-L-lysine has some antitumor effects of its own (Arnold et al., 1979) and could be used for the synthesis of a prodrug (Shen and Ryser, 1979). Poly-L-aspartic acid was used by Zunino et al. (1982) as a carrier for daunorubicin. Poly-L-glutamic acid has been used as a biodegradable drug delivery system (Zupon et al., 1983; Amoss et al., 1974; Kato et al., 1984), and also in combination with an antibody as a homing device (Rowland et al., 1975; Wilchek, 1979). Previous reseach in this laboratory indicated that a poly-L-glutamic acid–mitomycin C conjugate may have a greater effect against certain types of tumors than the parent compound (Kato et al., 1982).

Therefore, some physicochemical and antitumor characteristics of this poly-L-glutamic acid–mitomycin C conjugate (mol. wt. 11,000) were assayed. In order to get more information about the effects of charge, molecular weight and side-chain (spacer), conjugates of poly-L-lysine (PLY, mol. wt. 13,000), poly-L-aspartic acid (PAA, mol. wt. 14,000) and poly-L-glutamic acid (PGA, mol. wt. 60,000) were also synthesized and investigated.

Materials and Methods

Chemicals

Mitomycin C was kindly supplied by the Kyowa Hakko. Poly-L-glutamic acid, sodium salt (PGA 11, mol. wt. 11,000 and PGA 60, mol. wt. 60,000), poly-L-aspartic acid, sodium salt (PAA, mol. wt. 14,000) and poly-L-lysine hydrobromide (PLY, mol. wt. 13,000) were purchased from the Sigma Chemicals, U.S.A. The average molecular weights were determined by viscosity measurements.

Synthesis of the poly-L-glutamic acid–mitomycin C conjugates: PGA-M 11 and PGA-M 60

The conjugates of PGA and MMC were synthesized by reacting PGA with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and MMC in 10 ml distilled water, with a ratio of PGA 11 : EDC : MMC = 100 mg : 175 mg : 30 mg and PGA 60 : EDC : MMC = 100 mg : 300 mg : 27 mg. The reaction mixture was maintained for 4 h at room temperature at a pH between 5 and 6.

Synthesis of poly-L-aspartic acid–mitomycin C conjugate: PAA-M

The free amino-groups of PAA were masked by reacting 100 mg PAA with 70 mg succinic anhydride in isotonic phosphate buffer, pH 7.5. The reaction product was purified by gel filtration and concentrated by ultrafiltration. The purified PAA was conjugated with 20 mg MMC by reaction with 100 mg EDC for 3 h, at room temperature. The pH was maintained between 5 and 6.

Synthesis of poly-L-lysine–mitomycin C conjugates: PLY-SA-M and PLY-GA-M

The spacers, succinic acid and glutaric acid, were coupled to PLY by reacting 20 mg succinic anhydride or 22 mg glutaric anhydride to 100 mg PLY in 5 ml phosphate buffer, pH 7.5. The product was isolated by ultrafiltration and was allowed to react with 20 mg MMC and 50 mg EDC for 4 h at room temperature at pH 5–6.

Isolation of the conjugates

The conjugates were isolated by filtration through an ultrafilter (UK-10,size 43; Toyo, Japan) under high nitrogen pressure. The conjugates were freeze-dried after washing with distilled water. For the animal experiments, the conjugates were prepared in saline solution and injected i.p. at 0.1 ml/10 g body weight. The doses of the conjugates were expressed as the amount of parent MMC in the conjugates (equivalents of MMC).

In vitro release

The release of MMC from its conjugates was determined with a dialysis system. Cellulose Dialyzer Tubing (mol. wt. cutoff 8000, Nakarai Chemicals, Japan) containing 5 ml of pH 7.4 isotonic phosphate buffer with conjugate was immersed in 50 ml of the same buffer (in duplo). The solutions were maintained at 37°C and were shaken continuously. At fixed time intervals, samples of 1 ml were taken from the outer medium and replaced with 1 ml of the buffer. The release rate constant and the release half-life were calculated by the least-squares method. A correction was made for the dilution.

Molecular size

A Sephadex G-75 column (2.3 × 28 cm) was employed for molecular size determination. Isotonic phosphate buffer, pH 7.4, was used as the elution fluid. The flow rate was kept at 15 ml/h and fractions of 2.5 ml were collected automatically for analysis. For each sample, the K_{av} value was calculated from the following

equation:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e = elution volume of the sample; V_0 = column void volume; and V_t = total bed volume. The Stokes' radii of the samples were determined from the calibration curves obtained from K_{av} values of 4 kinds of protein of known molecular radii and molecular weight (Gel Filtration Kit, Pharmacia Fine Chemicals, Sweden).

Molecular charge

The molecular charge was estimated with DEAE-Sephadex A-50 anion exchanger and CM-Sephadex C-50 cation exchanger (Pharmacia Fine Chemicals, Sweden). Several buffers in saline were used in a concentration of 20 mmol/l: 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol (Bistris; pH 5.0 and 6.0); N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (Hepes; pH 7.2); N-2-hydroxyethyl-piperazine-N′-3-propanesulfonic acid (EPPS; pH 8.0); Tris(hydroxymethyl) aminoethane (Tris; pH 9.0 and 10.0); 3-cyclohexylaminopropanesulfonic acid (CAPS; pH 11.0). An ion exchanger (25 mg) was suspended in 5 ml buffered saline and shaken with 0.5 ml conjugate solution of 160 μg equivalent MMC/ml. After 5–10 min, the samples were centrifuged. The amount of MMC equivalent in the supernatant was measured spectrophotometrically at 364 nm and corrected for the absorbance of the buffer.

Analytical method

The amount of conjugated MMC as well as the free MMC was determined spectrophotometrically at 364 nm. The polymers were detected at 210 nm.

In vitro cell assay: continuous exposure

Several doses of MMC or conjugates were added to 1 ml growth medium with 1×10^5 mouse leukemia L1210 cells (in triplo). The growth medium consisted of RPMI 1640 medium with 15% fetal bovine serum. After 3 days of incubation at 37°C in 95% air and 5% CO_2, the cells were counted with a Bürker-Turk hemocytometer. The growth inhibition was calculated as the ratio of the number of cells in an untreated group minus the number of surviving cells in a treated group and then divided by the number of cells in the untreated group.

In vitro cell assay: 1 h exposure

Several doses of MMC or conjugate were added to 3 ml Hanks' solution containing 3×10^5 mouse leukemia L1210 cells. These solutions were incubated at 37°C. After 1 h, the cells were isolated by centrifugation and washed twice with 3 ml Hanks' solution. The remaining cells were suspended in 3 ml growth medium and divided over 3 plates. The cells were counted after 4 days of incubation at 37°C. The growth inhibition was expressed in the same way as by the continuous exposure experiment.

Cellular binding in vitro

Ehrlich Ascites Carcinoma cells (1×10^7) were incubated with 10 μg/ml MMC

equivalent, in vitro at 37°C. After several time intervals the solution was freed from cells by centrifugation. The amount of MMC equivalent in the supernatant was measured spectrophotometrically and used for the calculation of the fraction, bound to the EAC cells.

Animal experiments

Male DBA/2 mice and male BDF1 hybrid mice (C57Bl/6, female × DBA/2, male) were obtained from Shizuoka Agricultural Co-operative Association for Laboratory Animals, Shizuoka, Japan. The animals were kept on the breeding diet NMF (Oriental Yeast, Japan) with water available ad libitum, in a room maintained at 23 ± 1°C and a relative humidity of 55 ± 5%. Mouse leukemia P388 and L1210 were maintained by weekly transplantation of the tumor cells into the peritoneal cavity of male DBA/2 mice.

Evaluation of antitumor activity in vivo

BDF1 mice weighing 18–20 g were inoculated with 1×10^6 P388 mouse leukemia cells, intraperitoneally. Chemotherapy was given intraperitoneally, 24 h after inoculation. The activities of the compounds were indicated as the increase in life span (ILS %), calculated as the mean survival time of the treated group divided by that of the control group, multiplied by 100 and then minus 100. The observation period was 60 days.

Results

Synthesis of MMC conjugates

The conditions for the synthesis of the conjugates were chosen in such a way that cross-linking and formation of an insoluble product was prevented. The expected structures of MMC and the conjugates are shown in Fig. 1. The contents of MMC in the conjugates were fairly high and corresponded to a reasonable degree of substitution. Higher degrees of substitution could be obtained but this also increased the amount of insoluble products, due to the required amount of EDC.

The in vitro release is shown in Fig. 2. All conjugates exhibited monoexponential liberation of MMC. The release rates showed great differences (Table 1). PGA-M 11 ($t_{1/2}$ = 36.2 h) liberated MMC at the slowest rate among them. PGA-M 60 ($t_{1/2}$ = 12.7 h) and PLY-GA-M ($t_{1/2}$ = 12.5 h) had almost the same release rate. More rapid release rates were shown by PAA-M ($t_{1/2}$ = 5.14 h) and PLY-SA-M ($t_{1/2}$ = 6.04 h). The difference between PGA-M 11 and PGA-M 60 could be explained by difference in substitution ratio, which leads to change in microenvironment of the conjugate.

Gel filtration showed that MMC was coupled to the polyamino acid back-bone and that the conjugates did not contain significant amounts of low molecular weight contaminations.

Molecular size estimations

The molecular sizes of the polyamino acids and the conjugates were determined by the gel filtration method (Fig. 3). The gel filtration patterns of PGA 11 showed a

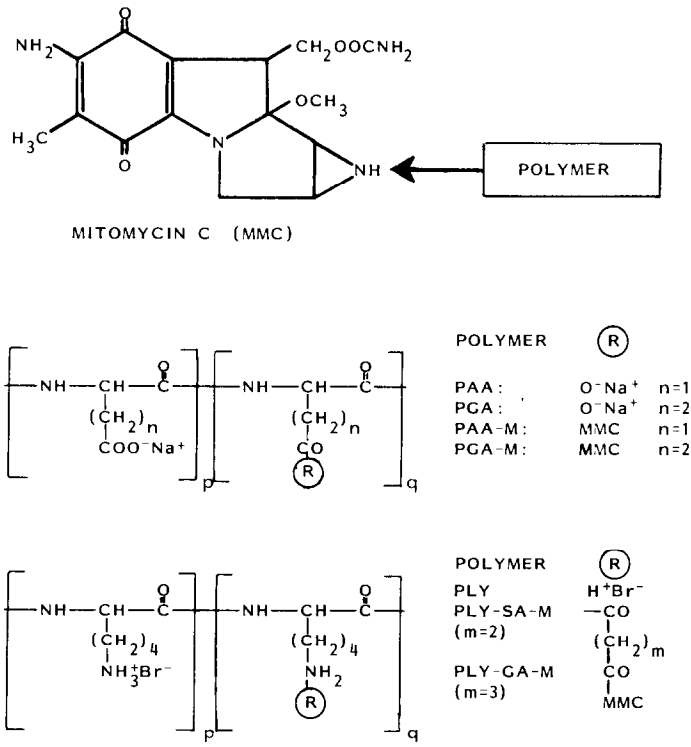

Fig. 1. Structures of mitomycin C and polyamino acid–mitomycin C conjugates.

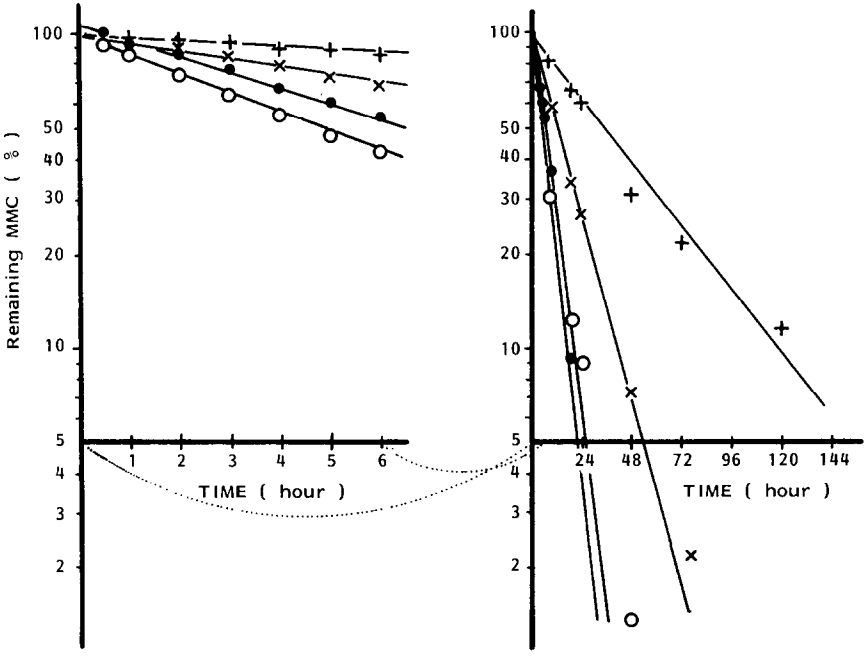

Fig. 2. In vitro release of mitomycin C from polyamino acid–mitomycin C conjugates. +, PGA-M 11; ×, PGA-M 60; ○, PAA-M; ●, PLY-SA-M. The data are means of two experiments and are shown as semi-logarithmic plots of percent remaining (bound) mitomycin C in the conjugates versus the incubation time. Both plots show the same data, but on a different time scale.

TABLE 1

MITOMYCIN C CONTENT AND THE RELEASE RATE OF THE POLYAMINO ACID CONJUGATES

Conjugate	MMC content (% w/w)	Substitution * ratio (units/MMC mol.)	Release rate constant (1/h)	$T_{1/2}$ rel. (h)
PGA-M 11	23	7 GA:1 MMC	0.0191	36.2
PGA-M 60	18	10 GA:1 MMC	0.055	12.7
PAA-M	13	16 AA:1 MMC	0.135	5.14
PLY-SA-M	14	10 LY:1 MMC	0.115	6.04
PLY-GA-M	14	10 LY:1 MMC	0.055	12.5

* Given are the number of polymer units per substituted MMC molecule. GA, glutamic acid; AA, aspartic acid; LY, lysine; PGA-M 11, polyglutamic acid–mitomycin C (mol. wt. 11,000); PGA-M 60, polyglutamic acid–mitomycin C (mol. wt. 60,000); PAA-M, polyaspartic acid–mitomycin C; PLY-SA-M and PLY-GA-M, polylysine-mitomycin C conjugate with, respectively, succinic acid and glutaric acid as spacer.

broad peak with 93% of the PGA 11 between the effective molecular sizes of 8.9 Å and 34.9 Å (values at 50% peak height), with the main peak at 23.5 Å (Table 2). The molecular sizes calculated from the given molecular weights (2000–15,000, peak 11,000) are 6.0–16.8 Å, peak 14.4 Å. Apparently, the effective molecular size of PGA

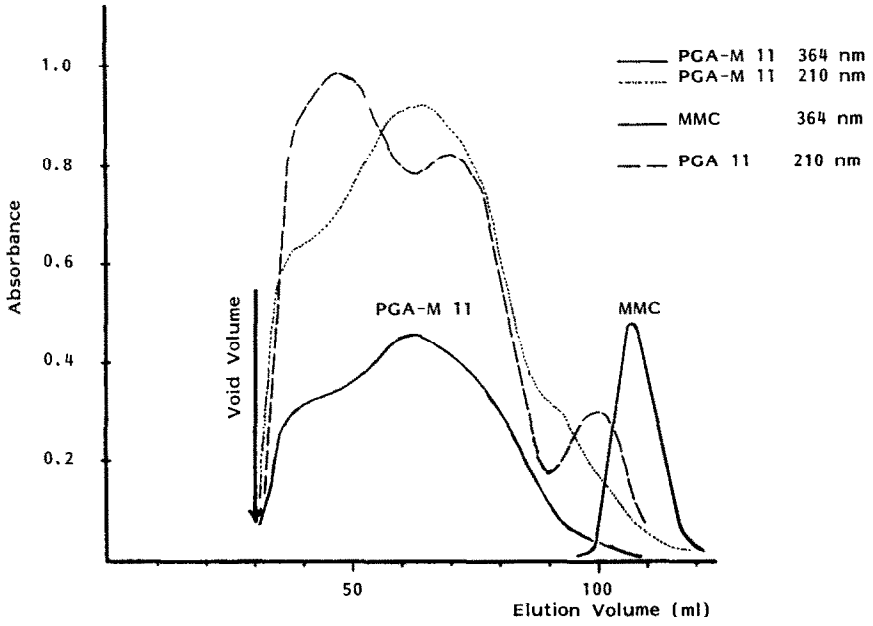

Fig. 3. Gel filtration patterns of mitomycin C, poly-L-glutamic acid and poly-L-glutamic acid–mitomycin C conjugate. ———, MMC; — — —, PGA 11; ———, ------, PGA-M 11. Chromatography was carried out on a Sephadex G-75 column (2.3×28 cm) with isotonic phosphate buffer, pH 7.4, at room temperature. Fractions of 2.5 ml were collected automatically for determination.

TABLE 2

MOLECULAR SIZES AS ESTIMATED BY GEL FILTRATION CHROMATOGRAPHY

Compound	Molecular * weight ($\times 10^3$)	K_{av} value	Estimated effective radius (Å)
PGA 11	11	0.220	23.5
PGA-M 11	–	0.399	15.0
PGA 60	60	0.030	37.8
PGA-M 60	–	0.350	17.0
PAA	14	0.066	34.6
PAA-M	–	0.139	28.8
PLY	13	0.484	12.2
PLY-SA-M	–	0.474	12.5
PLY-GA-M	–	0.530	10.8

* Molecular weight determined by viscosity measurement, as given by the manufacturer.

11 is larger than that of a comparable ideal molecule. The peak of the conjugate (PGA 11) was shifted towards a smaller effective radius (15.0 Å). PAA had a similar behaviour (peak PAA 34.6 Å vs PAA-M 28.8 Å). This shift was great in PGA 60 (from 37.8 Å to 17.0 Å). The PLY conjugates seemed to behave as ideal molecules.

Molecular charge

The experiments indicated (Fig. 4) that MMC and PGA-M 60 were neutral in the pH range 5–11. PGA-M 11 and PAA-M were adsorbed by the anion exchanger in the pH interval between 5.0 and 10.0. The PGA-M 11 and PAA-M conjugates acted as negatively charged molecules. The reduction of the adsorbed PGA-M 11 by the anion exchanger at pH 5.0 and 6.0 indicated that the pK_a value of the γ-carboxyl group was of that magnitude. PAA-M was more acidic. The PLY conjugates

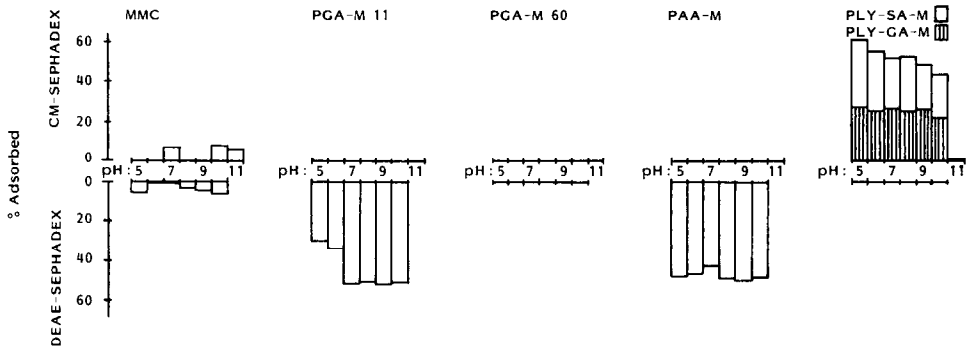

Fig. 4. Adsorption to CM- and DEAE-Sephadex cation, respectively, anion exchanger for several pH values. The binding percentage was calculated from the absorbance at 364 nm of the remaining amount of MMC (-conjugate) in the solutions and a standard. A correction was made for the absorbance of the buffers.

possessed a positive charge in the pH interval 5–10. The reduction in the adsorption at pH 10–11 indicated the pK_a value of the ϵ-amino groups. The cause of the neutral behaviour of PGA-M 60 is not clear but might be linked to the unexpected results obtained by gel filtration and the release rate. PGA-M 60 did not act as a larger molecular weight type of polyglutamic acid, compared to PGA-M 11.

Cellular ad-/absorption in vitro

The amount of MMC and MMC-conjugates bound to EAC cells after 15, 60 and 180 min are given in Fig. 5. Only PLY-SA-M showed significant binding to the cells.

Effects of MMC and its conjugates in vitro

The effects of continuous exposure of leukemia L1210 cells to the conjugates are

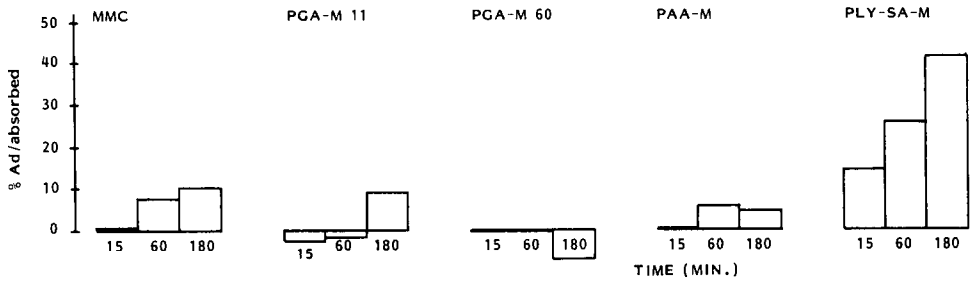

Fig. 5. Binding of mitomycin C and its polyamino acid conjugates to Ehrlich Ascites Carcinoma cells in vitro. 1×10^7 cells/ml were incubated with 10 μg equivalent MMC/ml for several time intervals. The ad-/absorption percentage was calculated from the absorbance of the remaining amount of MMC (-conjugate) in the solutions and a standard solution. A correction was made for the absorbance caused by the incubation with the cells.

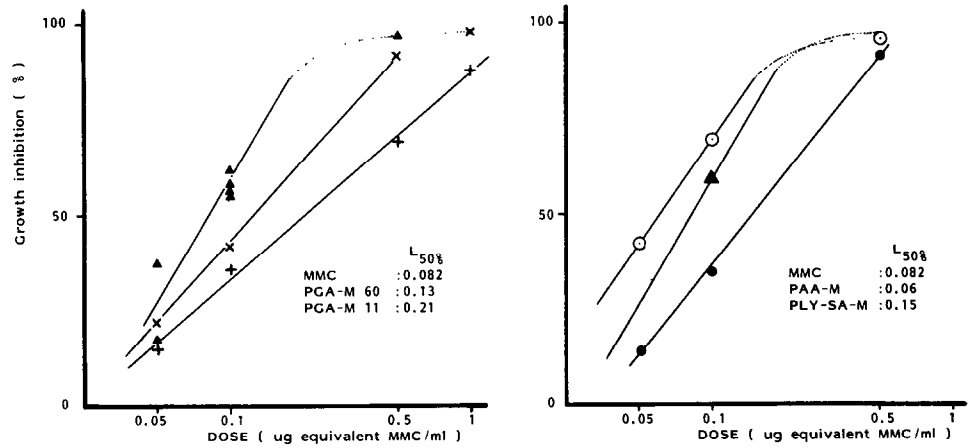

Fig. 6. Effect of continuous exposure of mouse L1210 leukemia to mytomycin C and its polyamino acid conjugates in vitro. ▲, MMC; +, PGA-M 11; ×, PGA-M 60; ⊙, PAA-M; ●, PLY-SA-M. The mean values of several experiments are shown as semi-logarithmic plots of % growth inhibition compared to untreated cells versus the dose. Effect was measured after 3 days of incubation.

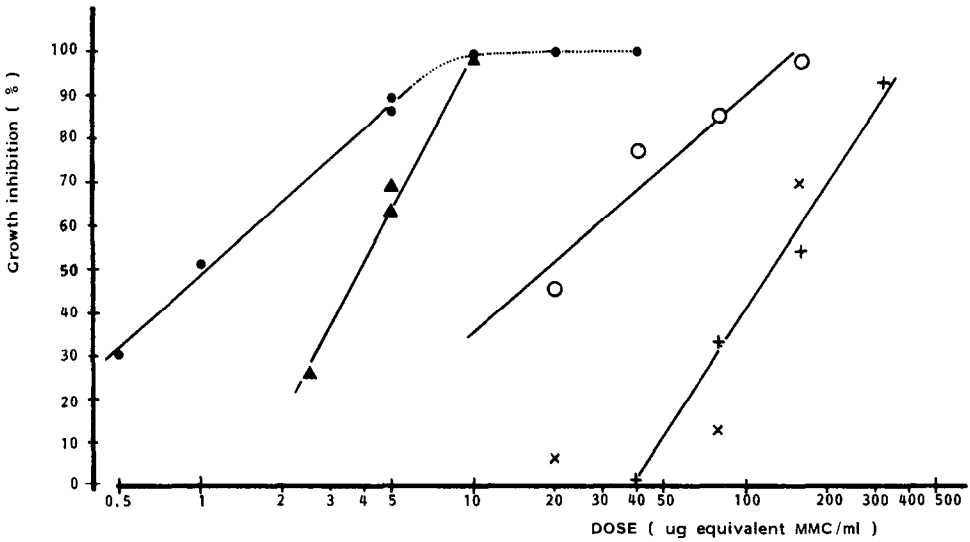

Fig. 7. Effect of 1 h exposure of mouse L1210 leukemia to mitomycin C and its polyamino acid conjugates in vitro. ▲, MMC; +, PGA-M 11; ×, PGA-M 60; ○, PAA-M; ●, PLY-SA-M. The effects were assayed after 4 days of incubation in growth medium. The mean values of several experiments are shown as semi-logarithmic plots of percentage growth inhibition compared to a standard, versus the dose.

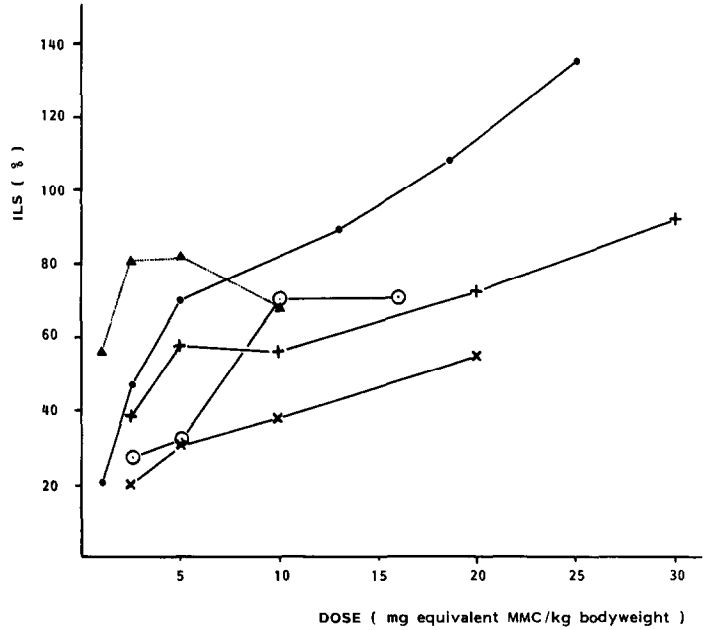

Fig. 8. Effects of mitomycin C and polyamino acid–mitomycin C conjugates on the survival time of mice bearing P388 leukemia. ▲, MMC; +, PGA-M 11; ×, PGA-M 60; ⊙, PAA-M; ●, PLY-SA-M. Each point represents the mean value of six mice. The mean survival time of the control group was 9.3 days. Three out of six mice treated with 10 mg/kg MMC and two out of six mice treated with 15 mg/kg PAA-M died early, due to severe toxicity.

similar to the effect of MMC (Fig. 6). But after 1 hr exposure of the leukemia cells (Fig. 7), the effects of the conjugates were much smaller, corresponding to their slow release rates. An exception was PLY-SA-M which showed greater effect than MMC.

Effects of MMC and its conjugates in vivo

The effects of several doses of MMC or its conjugates on the increase in the life span of mice bearing P388 leukemia were estimated (Fig. 8). The optimal dose of MMC was 5 mg/kg. Mice died from the toxicity of the drug at a dose of 10 mg/kg. Toxicity is obvious because a remarkable weight reduction was observed. The results showed that only the PAA-M conjugate showed some toxicity at a dose of 15 mg/kg. Its toxicity was only slightly less than MMC. For PGA-M 60, PLY-SA-M and PGA-M 11, no toxicity was observed at doses of, respectively, 4, 5 and 6 times the optimum MMC dose, while especially in the case of PLY-SA-M a better effect was observed than MMC at the high doses. PGA-M 60 was less effective than MMC and PGA-M 11.

Discussion

Mitomycin C has proven to be a potent antitumor antibiotic against a variety of human malignancies. However, its application has been limited by the severe bone marrow depression and gastrointestinal damage observed during its use. Therefore it is desirable to alter the localization and accessibility of the drug, such that the effect of the drug at the desired site of action is enhanced relative to effects at sites at which drug effects are unwanted. A macromolecular drug carrier system may accomplish this. First PGA-M 11, for example, and later PGA-M 60, PAA-M and PLY-SA-M were synthesized in order to make a latent form of MMC with lower toxicity than the parent drug.

Gel filtration and the release rate of the drug showed that covalent coupling of MMC and the polyamino acids were obtained. The in vitro cell assays indicated that the conjugates acted as latent prodrugs (effects of 1 h exposure vs continuous exposure) and that it was effective only after the release of MMC. The in vivo experiments with mice bearing P388 leukemia showed a decreased toxicity of the conjugates compared to MMC. The ion exchange experiment and the cellular binding showed that no complication of the antitumor effect of the PGA and PAA conjugates due to the interaction of the macromolecule with the (negative) cell membrane would be expected. The capability of polylysine with a cationic charge to bind to cells like L1210 leukemia and EAC cells could be used to obtain some drug targeting. The high effect of the PLY-SA-M conjugate after 1 h exposure in vitro could be a result of this. Since the release of MMC from the conjugates is independent from, for example, endocytosis (Hashida et al., 1983), a different type of action can be expected than that described by Arnold et al. (1983) against leukemia L1210. The relatively large effective size of the PGA and PAA conjugates might prove to be useful in the development of an immobile, but soluble, and biodegradable carrier for MMC.

In the case of the development of new polymeric carriers, the antigenicity of the polymer should be considered. In this investigation, only a small amount of polymer was required for chemotherapy, because the conjugates had high contents of MMC and MMC had remarkable antitumor activity even at low doses. Furthermore, the polyamino acids used had relatively low molecular weights. Therefore, the antigenicity of the conjugate could be considered to be low compared to general proteins such as albumin. But further studies are necessary.

It is shown that strongly negatively charged macromolecules are able to induce interferon production and increase the immune response (Ottenbrite et al., 1983; Przybylski et al., 1978). Therefore, the combination of such a polymer with the cytostatic antibiotic MMC is interesting. In this regard, it is interesting to see that PAA-M and PGA-M 11 (negative charge) are more effective than the neutral PGA-M 60 at similar doses. But more research should be done to confirm this for these anionic polyamino acids. Combining the results of PLY-SA-M and PGA-M 11, a polylysine–mitomycin C conjugate was synthesized with glutaric acid as spacer (PLY-GA-M) in order to obtain a positively charged molecule with a slower release rate. The results of the release rate and charge measurements were added to the other results. Also, current research is aimed at the development of a MMC-PGA conjugate with antibodies.

References

Amoss, M.S. jr., Monahan, M.W. and Verlaner, M.S., A long-acting polymer-coupled LRF analog. J. Clin. Endocr. Metab., 39 (1974) 187–190.

Arnold. L.J., Dagan, A., Gutheil, J. and Kaplan, N.O., Antineoplastic activity of poly(L-lysine) with some ascites tumor cells. Proc. Natl. Acad. Sci. U.S.A., 76 (1979) 3246–3250.

Arnold, L.J., Dugan, A. and Kaplan, N.O., Poly(L-lysine) as an antineoplastic agent and tumor-specific drug carrier. In Goldberg, E.P. (Ed.), Targeted Drugs, John Wiley, New York, 1983, pp. 89–112.

Bradner, W.T., In vivo evaluation of analogs. In Carter, S.K. and Crooke, S.T. (Eds.), Mitomycin C: Current Status and New Developments, Academic Press, New York, 1979, pp. 33–40.

Hashida, M., Kato, A., Kojima, T., Muranishi, S., Sezaki, H., Tanigawa, N., Satomura, K. and Hikasa, Y., Antitumor activity of mitomycin C–dextran conjugate against various murine tumors. Gann, 72 (1981) 226–234.

Hashida, M., Takakura, Y., Matsumoto, S. Sasaki, H., Kato, A., Kojima, T., Muranishi, S. and Sezaki, H., Regeneration characteristics of mitomycin C–dextran conjugate in relation to its activity. Chem. Pharm. Bull., 31 (1983) 2055–2063.

Hashida, M., Kato, A., Takakura, Y. and Sezaki, H., Disposition and pharmacokinetics of a polymeric prodrug of mitomycin C, mitomycin C–dextran conjugate, in the rat. Drug Metab. Dispos., 12 (1984) 492–499.

Kato, A., Takakura, Y., Hashida, M. Kimura, T. and Sezaki, H., Physico-chemical and antitumor characteristics of high molecular weight prodrugs of mitomycin C. Chem. Pharm. Bull. 30 (1982) 2951–2957.

Kato, Y., Saito, M., Fukushima, H., Takeda, Y. and Hara, T., Antitumor activity of 1-β-D-arabinofuranocylcytosine conjugated with polyglutamic acid and its derivatives. Cancer Res., 44 (1984) 25–44.

Kojima, T., Hashida, M., Muranishi, S. and Sezaki, H., Mitomycin C–dextran conjugate: a novel high molecular weight pro-drug of mitomycin C. J. Pharm. Pharmacol., 32 (1980) 30–34.

Ottenbrite, R.M., Kuus, K. and Kaplan, A.M., Characteristic biological effects of anionic polymers. In Chiellini, E. and Giusti, P. (Eds.), Polymers in Medicine (Biomedical and Pharmacological Applications), Plenum Press, New York and London, 1983, pp. 3–22.

Poznanski, M.J. and Cleland, L.G., Biological macromolecules as carriers of drugs and enzymes. In Juliano, R.L. (Ed.), Drug Delivery Systems, Oxford University Press, New York and Oxford, 1980, pp. 253–315.

Przybylski, M., Zaharko, D.S., Chirigos, M.A., Adamson, R.H., Schultz, R.M. and Ringsdorf, H., Divema-methotrexate: immune-adjuvant role of polymeric carriers linked to antitumor agents. Cancer Treat. Rep., 62 (1978) 1837–1843.

Rowland, G.F., O'Neill, G.J. and Davies, D.A.L., Suppression of tumor growth in mice by a drug–antibody conjugate using a novel approach to linkage. Nature (Lond.), 255 (1975) 487–488.

Sezaki, H. and Hashida, M., Macromolecule-drug conjugates in targeted cancer chemotherapy. In CRC Critical Reviews in Therapeutic Drug Carrier System, CRC Press, Boca Raton, FL, 1984, in press.

Shen, W.C. and Ryser, H.J-P., Poly(L-lysine) and poly(D-lysine) conjugates of methotrexate: different inhibitory effect on drug resistant cells. Mol. Pharmacol., 16 (1979) 614–622.

Takakura, Y., Matsumoto, S., Hashida, M. and Sezaki, H., Enhanced lymphatic delivery of mitomycin C conjugated with dextran. Cancer Res., 44 (1984) in press.

Wilchek, M., Affinity therapy and polymer bound drugs. Makromol. Chem., Suppl. 2 (1979) 207–214.

Zunino, F., Giuliani, F., Sari, G., Dasdia, T. and Gambetta, R., Anti-tumor activity of daunorubicin linked to poly-L-aspartic acid. Int. J. Cancer, 30 (1982) 465–470.

Zupon, M.A., Fang, S.M., Christensen, J.M. and Petersen, R.V., In vivo release of norethindrone coupled to a biodegradable poly(α-amino acid) drug delivery system. J. Pharm. Sci., 72 (1983) 1323–1326.